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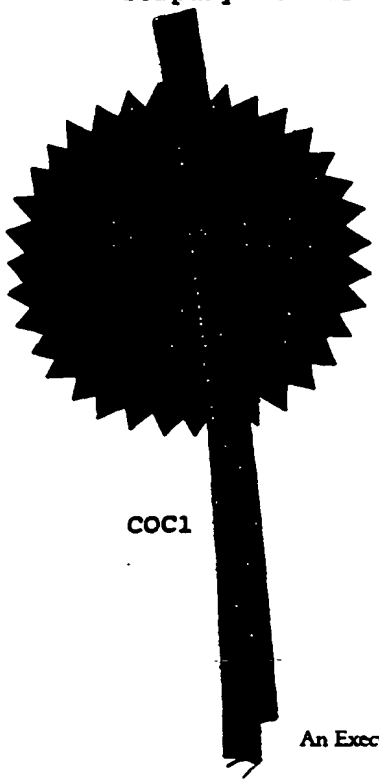

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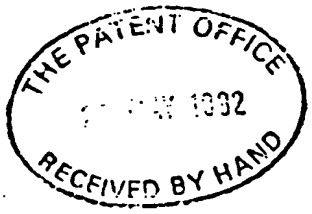

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27MAY92#00204096 PAT 1 77 UC 25.00

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Form 1/77

Patents Act 1977

❶ Title of invention

1 Please give the title of the invention: Monoclonal antibodies and their use.

❷ Applicant's details

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Agent's address Coomb House
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Agent's ADP number ~~02981701001~~ 1380001

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Continuation sheets for this Patents Form 1/77	no		
Claim(s)	1	Description	14
Abstract	1	Drawing(s)	8

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Monoclonal antibodies and their use

1. Background

As the result of the introduction of the hybridoma technology monoclonal antibodies (Mabs), mostly of mouse origin, have been made to many types of human cancer. Almost most of the markers defined by xenogeneic Mab are not strictly tumor specific but are differentiation antigens shared by tumors and certain normal and/or fetal tissues. Therefore, they are best referred to as tumor associated antigens (TAAs). Whether human tumor markers detected by xenogeneic Mabs are capable of evoking an antitumor response in cancer patients, and whether such antigens are indeed related to the response to autologous tumors in cancer patients, depends on the nature of the respective TAA and is still not fully understood. TAAs which are either naturally immunogenic in the syngeneic host or can be made immunogenic might potentially be used to induce antitumor immunity for therapeutic and possibly prophylactic benefit.

One approach towards manipulating the immune system is based on idiotypic interactions. The unique antigenic determinants in and around the antigen-combining site of an Ig molecule which make one antibody distinct from another are defined as idiotopes. The totality of all idiotopes present on the variable portion of a given antibody is referred to as its idiotype (id). The molecular structure of an idiotype has been localized to both the complementarity determining regions and the framework regions of the variable domain and is generally but not always contributed to by both the heavy and the light chains in specific association.

Idiotypes are serologically defined entities since injection of an antibody (often referred to as Ab1) into a syngeneic, allogeneic, or xenogeneic recipient induces the production of anti-idiotypic antibodies (often referred to as Ab2). Based on the assumption that idiotype/anti-idiotype interactions exist physiologically a receptor-based regulation of the immune system was postulated by Niels Jerne (Ann. Immunol. 125C, 373, 1974). His network theory views the immune system as a collection of Ig molecules and receptors on T lymphocytes, each capable of recognizing an antigenic determinant (epitope) through its combining site (paratope), and each capable of being recognized by other antibodies or cell-surface receptors of the system through the idiotopes that it displays. Many studies have indeed demonstrated that idiotypic and anti-idiotypic receptors are present on the surface of both B- and T-lymphocytes as well as on secreted antibodies.

When the binding between Ab1 and Ab2 is inhibited by the antigen to which Ab1 is directed, the idiotype is considered to be binding-site-related, since it involves a site on the antibody variable domain that is engaged in antigen recognition. Those idiotopes which conformationally

mimic an antigenic epitope are called the internal image of that epitope. Since both an Ab2 and an antigen bind to the relevant Ab1, they may share a similar three-dimensional conformation which represents the internal image of the given antigen.

Tumor associated antigens are often a part of "self" and evoke a very poor immune response in cancer patients. In contrast, internal image anti-idiotypic antibodies expressing three-dimensional shapes which resemble structural epitopes of the respective TAA are recognized as foreign molecules in the tumor bearing host. Therefore, the immune response raised by therapeutic or even prophylactic vaccination may cause antitumor immunity.

Monoclonal antibodies with specificity of BR55-2 (disclosed in e.g. Wistar EP 285 059, M.Blaszcyk-Thurin et al., J. Biol. Chem. 262 (1987) 372-379, or Z. Steplewski et al., Hybridoma 9 (1990) 201-210) define the Lewis Y6 antigen, a carbohydrate determinant selectively expressed on a majority of human solid tumors. Based on their properties antibodies BR55-2 can be used for passive immunotherapy of, basically, epithelial cancer.

The tumor associated Lewis Y oligosaccharide determinant which is also expressed during certain stages of embryonic development is almost not immunogenic by itself. However, monoclonal anti-idiotypic antibodies (Ab 2) against BR55-2 (Ab1) with internal image properties by resembling structural epitopes of the Lewis Y antigen may be useful for induction of a protective antitumor immunity.

The present invention comprises the generation, production and characterization of murine monoclonal internal image anti-idiotypic antibodies (Ab2) to monoclonal antibodies BR55-2 (Ab1) and the use of these anti-idiotypic Mabs for active immunization against Lewis Y antigen positive cancer.

2. Generation and characterization of murine monoclonal anti-idiotypic antibodies against the idiotype of antibodies BR55-2

In an attempt to minimize undesired anti-isotypic immune responses, the $F(ab')_2$-fragment of BR55-2, murine IgG3, was chosen for immunization. For the successful generation of murine anti-id Mabs against the idiotype of the murine Mab BR55-2, it is important to maximize the immunogenicity in order to raise an appropriate immune response in the syngeneic host. Therefore the $F(ab')_2$-fragment which is devoid of the Fc-part (cleavage and purification described in W 092/03165) was coupled to Keyhole Limpet Hemocyanin (KLH) as immunogenic carrier using the heterobifunctional linker N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP; Pharmacia) according to described methods (J. Carlsson et al., Biochem. J. 173, 723, 1978).

Balb/c mice were immunized with this BR55-2/murine IgG3-F(ab')$_2$-KLH-conjugate using Freund's complete adjuvant based on a typical protocol for the generation of murine Mabs. Following repeated immunizations the murine spleen cells were fused with the murine myeloma cell line SP2/0 (for experimental details see example 1).

For an appropriate selection of the cultured hybridoma cells a sequence of tests of their supernatants was performed. This selection was based on the following criteria:

a) Secretion rate of hybridomas by determination of the concentration of murine IgG in the supernatants (for experimental details see example 2). Cells producing high amounts of murine IgG were subcloned to single cell cultures.

b) Binding of selected supernatants to the F(ab')$_2$-fragment of BR55-2/murine IgG3 (for experimental details see example 3).

c) Inhibition of binding of BR55-2/murine IgG2a to Lewis Y antigen positive SKBR5 human breast cancer cells by selected supernatants (for experimental details see example 4).

The latter test is designed to be indicative for internal image properties of Ab2's. The murine IgG2a switch variant of BR55-2 was used for binding in order to minimize detection of Ab2's recognizing remaining constant regions of the F(ab')$_2$-fragment of BR55-2/murine IgG3 used for immunization. This test was performed in a quantitative manner based on the IgG concentration determined in test a) (example 2). Furthermore an excess of unspecific mouse IgG was added to this inhibition experiment in order to avoid any detection of Ab2's not specific for the idiotype of BR55-2.

Hybridomas were chosen which produce IgG with an inhibition capacity of more than 95% (inhibition of binding of BR55-2/murine IgG2a to the SKBR5 cell line).

Using the test procedures mentioned above six different hybridomas were finally selected and expanded (E4, C11, B3, B9, G6, G9). All six hybridomas produce murine IgG1 as detected by subtype ELISA using rabbit-anti-mouse IgG1/peroxidase (such as the reagent of Zymed).

All six hybridomas were cultured in roller flasks (37° C, 5% CO_2 in medium G; change of medium every 3 to 4 days) and the supernatants were collected for subsequent purification.

Each supernatant containing the respective anti-id BR55-2 Mab was purified using immunoaffinity chromatography. In general, affinity chromatography is based on the interaction between an immobilized ligand and the substance of interest. In the case of anti-idiotypic BR55-2 Mabs, the highly specific ligand for the affinity column is Mab BR55-2/murine IgG2a which binds the anti-idiotypic Mabs of choice (for experimental details see example 5).

The degree of purity of the isolated anti-id BR55-2 Mabs (E4, C11, B3, B9, G6, G9) was tested by analytical FPLC ion-exchange-chromatography, size-exclusion-chromatography, SDS-

PAGE and isoelectric focussing. Purity of all six anti-id BR55-2 Mabs was >95% (for experimental details see example 6; SDS-PAGE and isoelectric focussing are shown in figures 1 and 2).

The purified anti-id Mabs were quantitatively characterized by determination of their capacity to inhibit binding of BR55-2/murine IgG3 to the Lewis Y antigen positive SKBR5 cell line. All anti-id Mabs inhibit the binding of Ab1 to its antigen based on a 1:1 stoechiometry (for experimental details see example 7; representative results are shown in figure 3).

The final proof of the internal image properties of the anti-id BR55-2 Mabs described above and their use as surrogate for the Lewis Y carbohydrate antigen is based on their ability to generate an Ab3 response recognizing Lewis Y antigen positive cells in different species. According to the network theory of N. Jerne antibodies (Ab3) induced by immunization with internal image anti-id Mabs (Ab2) have a binding specificity similar to that of Ab1. Therefore the immune response evoked by immunization with anti-id BR55-2 Mabs should be specific for Lewis Y antigen positive tumor cells. Consequently protective antitumor immunity may be induced in man by immunization with anti-id BR55-2 Mabs.

For the investigation of the properties of an Ab3 response rabbits as well as rhesus monkeys were immunized with anti-id BR55-2 #E4 using aluminium hydroxide as adjuvant. This mild adjuvant is widely used in different vaccines for human use. As a negative control the animals were also immunized with the same amount of unspecific mouse IgG1. After four immunizations during 5 weeks sera were collected at week 9 (for experimental details see example 8). Binding of serum Ig to the Lewis Y antigen positive SKBR5 breast cancer cell line and to the Lewis Y antigen negative WM9 melanoma cell line was determined (for experimental details see examples 9 and 10).

Anti-id BR55-2 #E4 elicits a high titered humoral immune response both in rabbits and rhesus monkeys. Serum Ig of animals immunized with anti-id BR55-2 #E4 selectively binds to the Lewis Y antigen positive tumor cell line but not to the Lewis Y antigen negative WM9 cell line. In contrast, by immunization with unspecific mouse IgG1 almost no tumor cell binding serum Ig is detected. These results are summarized in table 1. In figures 4 and 5 representative Ig-binding curves obtained with pre- and immunesera of rabbits and rhesus monkeys in a cell-ELISA (SKBR5 cells) are shown. Binding of serum Ig of animals immunized with anti-id BR55-2 #E4 to SKBR5 cells can still be detected at a serum dilution of 1:10.000.

Two years after the initial immunization course the rhesus monkeys were boosted once with anti-id BR55-2 #E4 or unspecific mouse IgG1 (for experimental details see example 8). Bind-

ing of monkey serum Ig to the Lewis Y antigen positive SKBR5 breast cancer cell line was determined before as well as 1 week and 4 weeks after boost immunization.

In rhesus monkeys treated with anti-id BR55-2 #E4 two years after first immunization Lewis Y antigen positive tumor cell binding serum Ig still can be detected. 1 week following boost immunization tremendously increased titers of serum Ig specifically binding to Lewis Y antigen positive tumor cells are found in animals boosted with anti-id BR55-2 #E4. These results are summarized in Table 2.

A similar pattern of immunoreactivity of the different monkey sera before and after first immunization course as well as before and after the boost immunization can also be demonstrated by binding to a membrane preparation of Lewis Y antigen positive SKBR5 cells (for experimental details see example 11). The results are summarized in Table 3.

In conclusion, immunization with anti-id BR55-2 Mabs leads to a high titered immune response specific for Lewis Y antigen positive cancer cells. Thereby the internal image properties of these anti-id Mabs and their use as surrogate tumor antigen for therapeutic and prophylactic active immunization with the aim of induction of antitumor immunity in man is highlighted.

The following examples illustrate the invention. The abbreviations have the following meanings:

BSA:	bovine serum albumin
CDC:	complement dependent cytotoxicity
DMEM:	Dulbecco modified Eagle Medium
ELISA:	enzyme-linked immunosorbent assay
FCS:	fetal calf serum
Mab:	monolonal antibody
PBS:	phosphate-buffered saline
RPMI:	Rosewell Park Memorial Institute
SDS:	sodium dodecyl sulfate
KLH:	keyhole limpet hemocyanin
SPDP:	N-succinimidyl-3-(2-pyridyl-dithio-propionate)
PAGE:	polyacrylamide gel electrophoresis
IEF:	isoelectric focussing
PEG:	polyethyleneglycol

The materials referred to in the examples are as follows:

Microtiterplates: Immunoplates II (Nunc)

Cell lines: SKBR5: human breast cancer cell line
WM9: human melanoma cell line
SP2/0: mouse myeloma cell line

Medium A: RPMI 1640 + 2 g/l $NaHCO_3$
100 U/ml penicillin G
100 µg/ml streptomycin sulfate
4 mM glutamine
10 % FCS (heat-inactivated, γ-globulin-free)

Medium B: RPMI 1640 + 2 g/l $NaHCO_3$
100 U/ml penicillin G
100 µg/ml streptomycin sulfate
4 mM glutamine
5 % FCS (heat-inactivated)

Medium C: DMEM
10 % NCTC-135 (synthetic medium, Gibco)
1 % MEM non essential amino acids (Gibco)
0.5 % sodium pyruvate
0.5 % oxalacetic acid (Sigma)
20 % FCS (heat-inactivated)
4 mM glutamine
100 U/ml penicillin G
100 µg/ml streptomycin sulfate

Medium D: Medium C + 1.36 mg/l hypoxanthine
0.39 mg/l thymidine

Medium E: Medium D + 0.4 mg/l aminopterin

Medium F: Medium C + mouse thymocytes (thymocytes of one Balb/c mouse resuspended in 25 ml medium C)

Medium G: DMEM
10 % FCS (heat-inactivated)
4 mM glutamine
100 U/ml penicillin G
100 µg/ml streptomycin sulfate

PEG: polyethyleneglycol (MW = 3400)
1 g is dissolved in 1 ml DMEM

PBS deficient: 138.0 mM NaCl
1.5 mM KOH
2.7 mM KCl
6.5 mM Na_2HPO_4
pH 7.2

Coating buffer: 15 mM Na_2CO_3
35 mM $NaHCO_3$
3 mM NaN_3
pH 9.6

Staining buffer: 24.3 mM citric acid
51.4 mM Na_2HPO_4
pH 5.0

Washing buffer: 2% NaCl
0.2 % Triton X-100
in PBS deficient

Substrate solution: 40 mg o-phenylenediaminedihydrochloride
100 ml staining buffer
20 µl H_2O_2 30%

Binding buffer: 0.1 M Tris/HCl
0.2 M NaCl
pH 7.5

Elution buffer: 0.15 M glycine/HCl
0.2 M NaCl
pH 2.8

Coupling buffer: 0.1 M $NaHCO_3$
0.5 M NaCl
pH 8.0

In the following examples which illustrate the invention but in no way limit its scope references to temperature are in degrees celsius.

Example 1: Generation of anti-id BR55-2 #E4

1.1 Immunization of mice

Balb/c mice are immunized with each 100 µg of $F(ab')_2$-fragment of BR55-2/murine IgG3, coupled to KLH via SPDP as described (J. Carlsson et al., Biochem. J. 173, 723, 1978) by intraperitoneal injection in the following scheme:

day 0: 100 µg of conjugate (1 mg/ml in PBS def.) + 100 µl of Freund's complete adjuvant

day 7 and 28: 100 µg of conjugate (1 mg/ml in PBS def.) + 100 µl of Freund's incomplete adjuvant

On days 8, 9, 10 and 11 after primary immunization i.v. a total of 4 boost injections (each 100 µg of conjugate in 100 µl of PBS def.) are given.

On day 12 the spleens are taken out aseptically, suspended in PBS def. and washed thrice in PBS def.

1.2 Hybridization

These spleen cells are added to a suspension of SP2/0 cells in a ratio 1:1 and centrifuged at 900 g for 5 minutes. 1 ml of PEG-solution (37°) is added dropwise to the cell pellet within 1 minute and diluted with 1 ml of PBS def. (37°) within the next minute. 10 ml of medium C are added under gently rotation and the suspension is diluted to 50 ml with PBS def. The suspension is centrifuged at 800 g for 5 minutes, the pellet resuspended in medium D and the cells are transferred into the wells of a microtiterplate (Nunc 96) at a concentration of 2.5×10^5 cells/well. After overnight incubation at 37°/5% CO_2 100 µl/well of medium E are added. After 72 hours and then every four days the medium is replaced by medium D.

Example 2: Quantitative determination of mouse IgG in hybridoma supernatants

100 µl aliquots of rabbit-anti-mouse-IgG (such as the reagent of Nordic; 1:1000 in coating buffer) are added to the wells of microtiter plates, and incubated at 37° for 60 minutes.

The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. 100 µl aliquots of the hybridoma supernatants obtained after 2 weeks culture are added and the plates are incubated for 60 minutes at 37°. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (rabbit-anti-mouse-IgG/peroxidase such as the reagent of Dianova; 1:1000 in PBS/2% FCS) are added.

After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer.

100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Photometric extinction is measured at 492 nm (reference measurement 620 nm).

Example 3: Specific binding of hybridoma supernatant-IgG to BR55-2 F(ab')$_2$-fragment (ELISA)

Hybridomas producing sufficient mouse IgG (i.e. more as 10-fold optical density than the medium-blank) are subcloned to single cell culture in medium F and cultured in medium G for additional 2 weeks. The supernatants are tested as follows:

100 µl aliquots of F(ab')$_2$-fragment of BR55-2 (10 µg/ml; dilution in coating buffer) are added to the wells of microtiter plates, and incubated at 37° for 60 minutes.

The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. 100 µl of hybridoma supernatants are added and the plates are incubated for 60 minutes at 37°. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (rabbit-anti-mouse-IgG-Fc/peroxidase such as the reagent of Dianova; 1:1000 in PBS/2 % FCS) are added.

After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer.

100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Photometric extinction is measured at 492 nm (reference measurement 620 nm).

Example 4: Inhibition of binding of BR55-2/murine IgG2a to SKBR5 human breast cancer cells by hybridoma supernatant-IgG (cell ELISA)

All hybridoma supernatants which are positive in the above described assay are tested as follows:

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD; 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with 50 µl/well of a suspension of SKBR5 cells in medium B ($4x10^6$ cells/ml).

After removal of the supernatant the cells are fixed with 50 µl of glutardialdehyde/well (0.1% in physiological saline) for 5 minutes at room temperature, the supernatants are removed, the cells resuspended in 200 µl/well of PBS def./1% BSA/0.1% NaN_3 and left for 1 hour at room temperature.

Supernatants are removed and the plates are washed twice with 200 µl/well of PBS containing 0.05% Tween 20. Hybridoma supernatants adjusted to 1 µg/ml mouse IgG are preincubated with 10-fold excess of unspecific mouse IgG for 30 minutes at 37°. Then these samples are preincubated with 0.5 µg/ml of BR55-2/murine IgG2a for 30 minutes at 37°. 100 µl of this mixture are added to the cells and the plates are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl/well of ice-cold PBS containing 0.05 % Tween 20. 100 µl aliquots of peroxidase-conjugated antibody (rabbit-anti-mouse IgG2a/peroxidase such as the reagent of Zymed; 1:1000 in PBS def./2% FCS) are added. After incubation for 45 minutes at 37° the wells are washed thrice with the PBS/Tween 20 solution mentioned above and then 100 µl of the substrate solution are added to each well. After 5 minutes the colour development is stopped by addition of 50 µl aliquots of 4 N H_2SO_4/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (reference measurement 620 nm).

Example 5: Immunoaffinity purification of anti-id BR55-2 Mabs

5.1 Preparation of BR55-2/murine IgG2a Sepharose

10 g of freeze dried activated CH-Sepharose 4B is suspended in 1mM HCl, transferred to a sinter glass filter and washed with 2 l of 1 mM HCl for 15 minutes. The ligand (120 mg of BR55-2/murine IgG2a) dissolved in 50 ml of coupling buffer is mixed with the washed gel in a stoppered vessel and rotated end over end for one hour at room temperature. The gel is washed with coupling buffer and incubated for one hour with 50 ml of 1M ethanolamine for blocking of any remaining active groups. The affinity sorbent is then washed with three cycles of alternating pH. Each cycle consists of a wash at pH 4 (0.1 M acetate, 0.5 M NaCl) followed by a wash at pH 8 (0.1 M Tris, 0.5 M NaCl).

5.2 Isolation of the anti-id BR55-2 Mabs

The chromatography is performed at 4°. The column (BIO REX MP column, diameter 1.5 cm) is filled with Mab BR55-2/murine IgG2a Sepharose (volume 35 ml). The gel is washed with binding buffer and elution buffer. After equilibration with binding buffer conditioned medium containing anti-id BR55-2 is loaded onto the column at a flow rate of 15 ml/min. After elution of the breakthrough fraction, the bound anti-id BR55-2 is desorbed with elution buffer and neutralized immediately after desorption with 1 M Tris/HCl buffer, pH 7.5.

5.3 Concentration of the anti-id BR55-2 Mabs

Concentration of the eluted antibody solution (0.12 mg/ml) is performed in a stirred Amicon ultrafiltration cell using a PM 10 Diaflo membrane. The solute rejection for IgG is more than 98%, the final concentration of IgG amounts to 3.7 mg/ml.

Example 6: Characterization of purified anti-id BR55-2 Mabs

6.1 Ion-exchange-chromatography on Mono-Q

Column:	Mono-Q HR5/5 (Pharmacia)
Buffer A:	20 mM tri-ethanolamine, pH 7.7
Buffer B:	20 mM tri-ethanolamine, 1 M NaCl, pH 7.7
Flow rate:	1 ml/min
Detection:	UV 280 nm
Gradient:	linear 2%/min
Results:	> 95% purity found for all anti-id BR55-2 Mabs

6.2 High performance size-exclusion-chromatography

Column:	Zorbax GF250, 9.4 x 250 mm
Buffer :	Sodium phosphate 0.1 M, 0.2 M NaCl, pH 7.0
Flow rate:	1 ml/min
Detection:	UV 280 nm
Results:	> 95% purity found for all anti-id BR55-2 Mabs

6.3 SDS-PAGE

Experiments are performed both under reducing and non-reducing conditions according to the method of Laemmli using 10 % acrylamide gels (results are shown in Figure 1).

6.4 Isoelectric focussing

Analysis is performed with the Phast-system (Pharmacia) using a pH-gradient 3-9 (Phast gel IEF 3-9) and silver staining for detection of the protein bands (results are shown in figure 2).

Example 7: Binding of BR55-2/murine IgG3 to SKBR5 cell line (cell-ELISA) - Inhibition by anti-Id BR55-2 #E4

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD; 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with 50 µl/well of a suspension of SKBR5 cells in medium B ($4x10^6$ cells/ml). After removal of the supernatant the cells are fixed with 50 µl of glutardialdehyde/well (0.1 % in physiological saline) for 5 minutes at room temperature, the supernatants are removed, the cells resuspended in 200 µl/well of PBS def./1% BSA/0.1% NaN_3 and left for 1 hour at room temperature.

Supernatants are removed and the plates are washed twice with 200 µl/well of PBS containing 0.05 % Tween 20. Anti-id BR55-2 #E4 is diluted in PBS def. containing 2% FCS (10 to 0.5 µg/ml). To each of these dilutions 1 µg/ml of BR55-2/murine IgG3 is added. 100 µl of

this mixture are added to the cells and the plates are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl/well of ice-cold PBS containing 0.05 % Tween 20. 100 µl aliquots of peroxidase-conjugated antibody (rabbit anti-mouse IgG3/peroxidase such as the reagent of Zymed; 1:1000 in PBS def./2% FCS) are added. After incubation for 45 minutes at 37° the wells are washed thrice with the PBS/Tween 20 solution mentioned above and then 100 µl of the substrate solution are added to each well. After 5 minutes colour development is stopped by addition of 50 µl of 4 N H_2SO_4/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (reference measurement 620 nm).

Example 8: Immunization of rabbits and rhesus monkeys with anti-id BR55-2 #E4

8.1 Immunization of rabbits with anti-id BR55-2 #E4

Three female chinchilla rabbits are immunized by intradermal application of 300 µg of anti-id BR55-2 #E4 adsorbed on aluminium hydroxide (1 mg of antibody plus 3.3 mg of $Al(OH)_3$/ml PBS def.) on days 1, 8, 15 and 36. Three rabbits are immunized with the same amount of unspecific mouse IgG1 as negative control under the same conditions. Sera are collected before immunization and at week 9 after first immunization.

8.2 Immunization of rhesus monkeys with anti-id BR55-2 #E4

Three rhesus monkeys are immunized by subcutaneous (s.c.) application of 0.1 mg of anti-id BR55-2 #E4/kg adsorbed on aluminium hydroxide (1 mg of antibody plus 3.3 mg of $Al(OH)_3$/ml PBS def.) on days 1, 8, 15 and 36. Two rhesus monkeys are immunized with the same amount of unspecific mouse IgG1 as negative control under the same conditions. Sera are collected before immunization and at week 9 after first immunization.

Two years after first immunizations the same monkeys receive one s.c. boost injection with anti-id BR55-2 #E4 or unspecific mouse IgG1 respectively (same amount and formulation as above). Sera are collected before as well as 1 week and 4 weeks after boost immunization.

Example 9: Binding of rabbit serum Ig to SKBR5 cell line (cell-ELISA)

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD; 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with 50 µl/well of a suspension of SKBR5 cells in medium B ($4x10^6$ cells/ml). After removal of the supernatant the cells are fixed with 50 µl of glutardialdehyde/well (0.1% in physiological saline) for 5 minutes at room temperature, the supernatants are removed, the cells resuspended in 200 µl/well of PBS def./1% BSA/0.1% NaN_3 and left for 1 hour at room temperature.

Supernatants are removed and the plates are washed twice with 200 µl/well of PBS containing 0.05% Tween 20. 100 µl aliquots of rabbit sera in appropriate predilutions are added to the

cells and the plates are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl/well of ice-cold PBS containing 0.05% Tween 20. 100 µl aliquots of peroxidase-conjugated antibody (goat-anti-rabbit-Ig/peroxidase such as the reagent of Dianova; 1:1000 in PBS def./2% FCS) are added. After incubation for 45 minutes at 37° the wells are washed thrice with the PBS/Tween 20 solution mentioned above and then 100 µl of the substrate solution is added to each well. After 5 minutes colour development is stopped by addition of 50 µl of 4N H_2SO_4/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (reference measurement 620 nm).

Example 10: Binding of rhesus monkey serum Ig to SKBR5 cell line (cell-ELISA)

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD; 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with 50 µl/well of a suspension of SKBR5 cells in medium B (4×10^6 cells/ml). After removal of the supernatant the cells are fixed with 50 µl of glutardialdehyde/well (0.1% in physiological saline) for 5 minutes at room temperature, the supernatants are removed, the cells resuspended in 200 µl/well of PBS def./1% BSA/0.1% NaN_3 and left for 1 hour at room temperature.

Supernatants are removed and the plates are washed twice with 200 µl/well of PBS containing 0.05% Tween 20. 100 µl aliquots of rhesus monkey sera in appropriate predilutions are added to the cells and the plates are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl/well of ice-cold PBS containing 0.05% Tween 20. 100 µl aliquots of peroxidase-conjugated antibody (goat-anti-human-Ig/peroxidase such as the reagent of Chemicon & Co., 1:1000 in PBS def./2% FCS) are added. After incubation for 45 minutes at 37° the wells are washed thrice with the PBS/Tween 20 solution mentioned above and then 100 µl of the substrate solution are added to each well. After 5 minutes colour development is stopped by addition of 50 µl of 4 N H_2SO_4/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (reference measurement 620 nm).

Example 11: Binding of rhesus monkey serum Ig to a SKBR5-membrane preparation (ELISA)

Membranes of the SKBR5 human breast cancer cell line were prepared as described by D. Thom et. al., Biochem. J. 168, 187-194 (1977).

100 µl aliquots of this membrane preparation (10 µg/ml; dilution in coating buffer) are added to the wells of microtiter plates, and incubated at +4° overnight. The plates are washed 4 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. 100 µl aliquots of rhesus monkey sera in

appropriate dilutions in PBS def./2% FCS are added and the plates are incubated for 60 minutes at 37°. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (goat-anti-human-Ig/peroxidase such as the reagent of Chemicon & Co., 1:1000 in PBS/2% FCS) are added. After incubation for 30 minutes at 37° the plates are washed twice with washing buffer and twice with staining buffer. 100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Optical density (OD) is measured at 492 nm (reference measurement 620 nm).

On view of the above experimental results anti-idiotypic BR55-2 Mabs are thus indicated for active immunization against adenocarcinomas and similar tumors, e.g. breast-, colorectal-, gastric-, pancreatic-, prostate-, ovarian- and lung cancer including small cell lung cancer, particularly for therapeutic use in humans.

For the above-mentioned use the dosage will, of course, vary depending upon e.g. the compound employed, the subject patient's age, the stage of disease, the mode of administration or the treatment desired, and can be determined by the specialist in each individual situation. It will also vary when the antibodies are used in combination with other antitumor agents or immunomodulating compounds. Administration is e.g. parenteral by subcutaneous injection possibly together with a suited adjuvant like aluminium hydroxide. The administration may be combined with immunomediators like IL-2, CSF, INF and/or low molecular weight immunostimulants. The dosage administered is e.g. of from about 0.05 mg to about 10 mg of anti-id Mab as defined above. A typical administration schedule e.g. consists of 1 to 4 basic immunizations during 2 to 6 weeks and repeated boost immunizations e.g. every second month or on an indiviual basis after substantial decrease of Ab3 titers.

Claims:

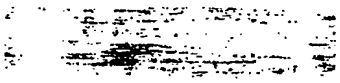
1. Monoclonal murine internal image anti-idiotypic antibodies (Ab2) to monoclonal antibodies BR55-2 (Ab1).

2. Process for the production of anti-idiotypic antibodies according to claim 1 which comprises immunizing mice with BR55-2/murine IgG3-F(ab')$_2$-KLH-conjugate, fusing the murine spleen cells with the murine myeloma cell line SP 2/0, selecting the cultured hybridoma cells which produce IgG with an inhibition capacity of more than 95% (inhibition of binding of BR55-2 murine IgG2a to the SKBR5 cell line), purifying and isolating the anti-idiotypic antibody.

3. Use of monoclonal anti-idiotypic antibodies as defined in claim 1 for immunization against adenocarcinomas and similar tumors and against small cell lung cancer.

4. A pharmaceutical composition which comprises an anti-idiotypic monoclonal antibody as defined in claim 1 together with a pharmaceutically acceptable adjuvant, carrier or diluent for immunization against adenocarcinomas and similar tumors and against small cell lung cancer.

5. A method of immunization against adenocarcinonas and similar tumors and against small cell lung cancer, which comprises administering to a subject in need of such a treatment an effective amount of an anti-idiotypic monoclonal antibody as defined in claim 1.



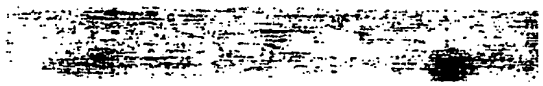


Figure (s) to accompany abstract

900-9639

Abstract:

Monoclonal murine internal image anti-idiotypic antibodies (Ab2) to monoclonal antibodies BR55-2 (Ab1), process for their production and their use for immunization against adenocarcinomas and similar tumors and small cell lung cancer.

SR/hz

Table 1

Immunization of rabbits and monkeys with anti-id BR55-2 #E4 or unspecific mouse IgG1

Relative titer increase at week 9 compared to 1:4 diluted preserum (Cell ELISA)

	Rabbit		Monkey	
	anti-id BR55-2	mouse IgG1	anti-id BR55-2	mouse IgG1
SKBR5 (Y^+)	1000	16	1000	8
WM9 (Y^-)	3	n.t.	25	n.t.

Table 2

Boost immunization of monkeys with anti-id BR55-2 #E4

Binding of monkey Ig to SKBR5 cells (Cell-ELISA)

Relative titer increase at week 9 compared to 1:4 diluted preserum

9 weeks after first course	2 years after first course	1 week after boost	4 week after boost
1000	100	1500	1000

Table 3

Boost immunization of monkeys with anti-id BR55-2 #E4 or unspecific mouse IgG1

Binding of monkey Ig to a SKBR5-membrane preparation (ELISA)

Relative titer increase at week 9 compared to 1:4 diluted preserum

	9 weeks after first course	2 years after first course	1 week after boost	4 weeks after boost
anti-id BR55-2	40	1	80	50
mIgG1	1	2	n.t.	2

Figure 1
SDS - PAGE

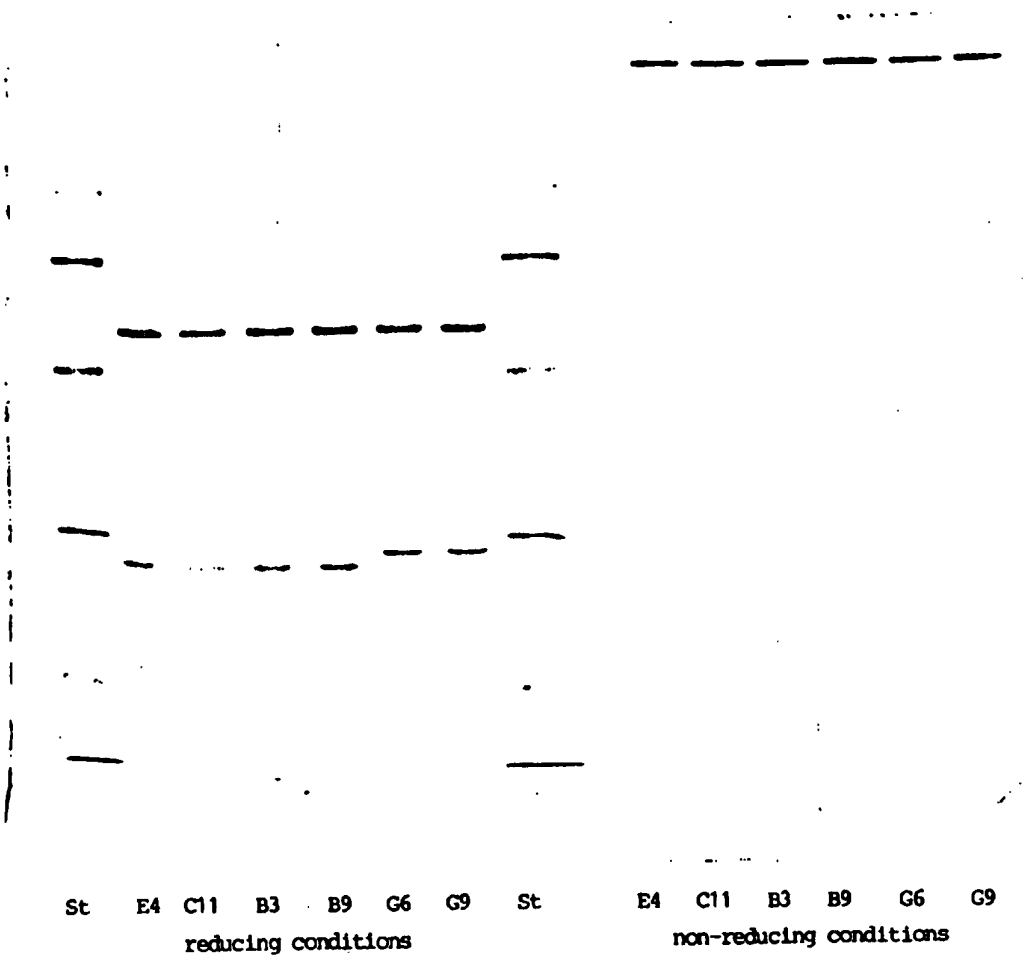
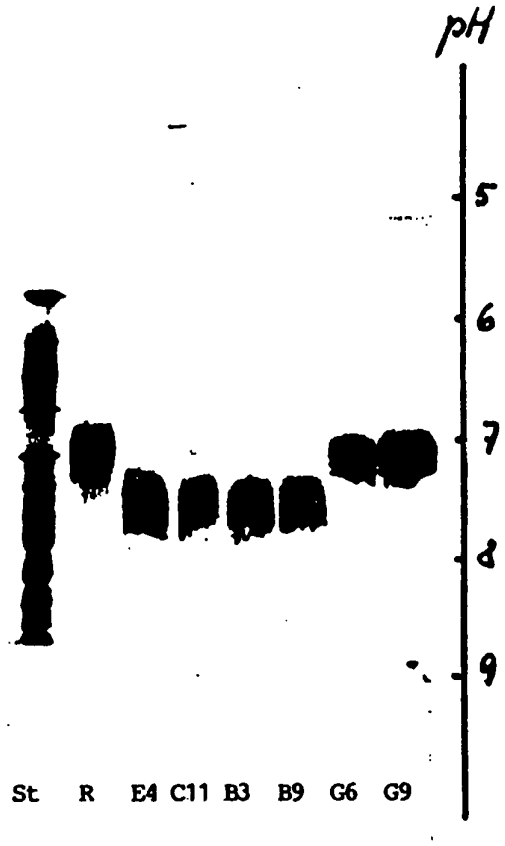
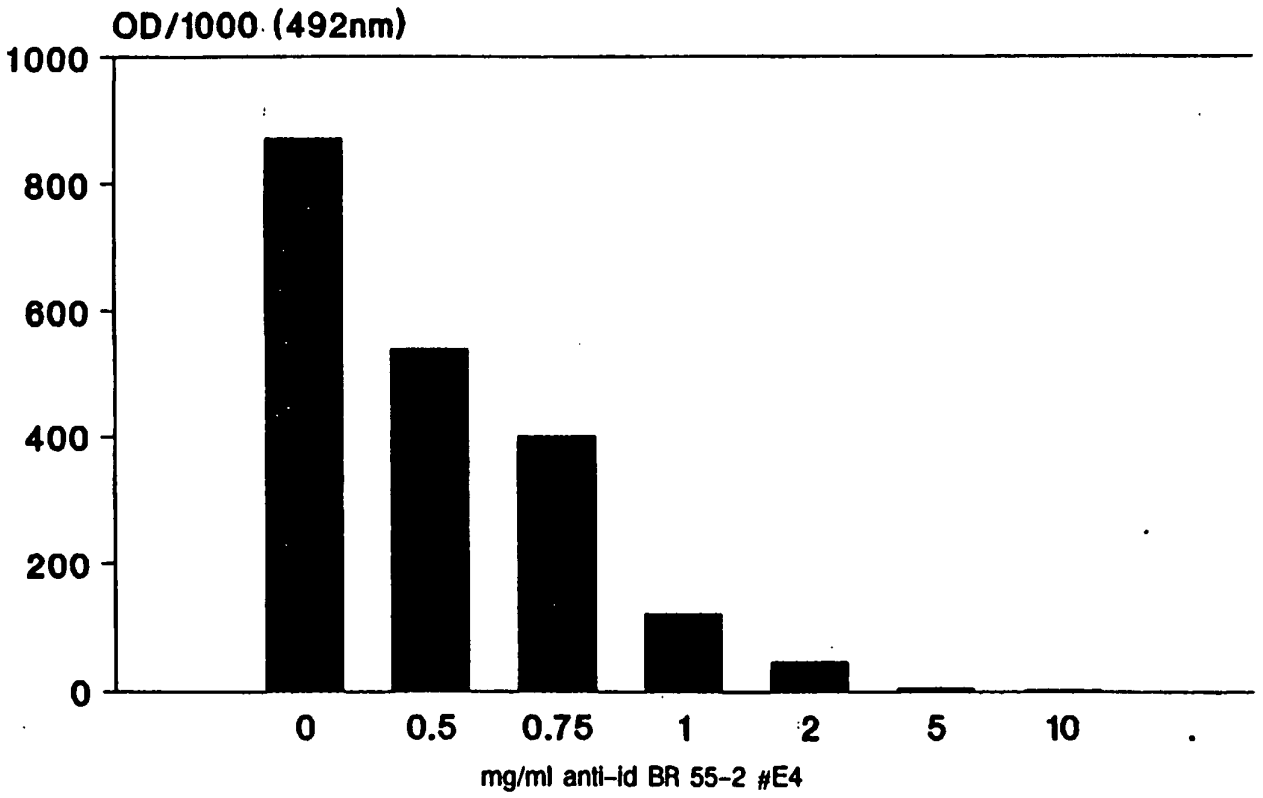


Figure 2
Isoelectric Focussing



St = Standard
R = Reference Mab
E4, C11, B3, B9, G6 and G9 = anti-id BR55-2 Mabs

Figure 3
Binding of BR55-2/murine IgG3 to SKBR5 cell line
Inhibition by anti-id BR55-2 #E4



BR 55-2/murine IgG3: 1 µg/ml

Figure 4
Binding of rabbit Ig to SKBR5 before and after immunization with anti-Id BR55-2 #E4 (Cell-ELISA)

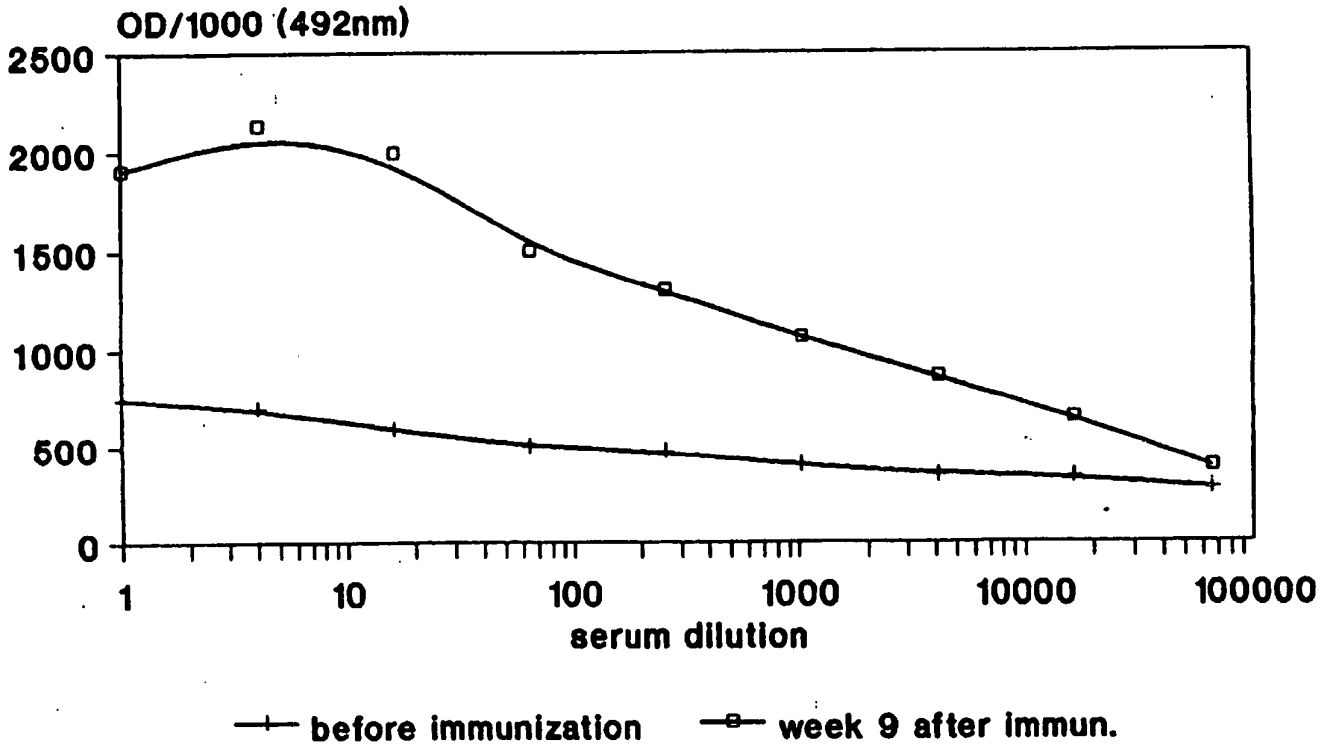


Figure 5
Binding of rhesus monkey Ig to SKBR5 before and after immunization with anti-Id BR55-2 #E4 (Cell-ELISA)